

# *Acta Medica Okayama*

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*Volume 63, Issue 5*

2009

*Article 3*

OCTOBER 2009

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## Age-dependent vulnerability to ischemia-reperfusion injury of cyanotic myocardium in a chronic hypoxic rat model

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# Age-dependent vulnerability to ischemia-reperfusion injury of cyanotic myocardium in a chronic hypoxic rat model

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## Abstract

This study evaluated the effects of chronic hypoxia from birth on the resistance of rat hearts to global ischemia, with special emphasis on the duration of hypoxia. Male Wistar rats were housed from birth for 4 weeks or 8 weeks either in a hypoxic environment (FiO<sub>2</sub>0.12) or in ambient air (8 animals for each group). Isolated rat hearts were perfused for 40 min with oxygenated Krebs-Henseleit buffer, subjected to 20 min global no-flow ischemia at 37, and then underwent 40 min of reperfusion. A non-elastic balloon was inserted into the left ventricle and inflated until the pre-ischemic LVEDP rose to 8mmHg. Cardiac function was measured before and after ischemia. The post-ischemic percent recovery of LVDP in hypoxic hearts was worse than in normoxic hearts (4 weeks:55±7 vs. 96±3%, p0.01; 8 weeks:40±5 vs. 92±4%, p0.01), and was worst in the 8-week-hypoxic hearts. Similarly, the percent recovery of dP/dt in the hypoxic hearts was lower than in the normoxic hearts (4 weeks:51±5 vs. 96±7%, p0.01; 8 weeks:31±6 vs. 92±7%, p0.01), and was lowest in the 8-week-hypoxic hearts. In conclusion, cyanotic myocardium revealed an age-dependent vulnerability to ischemia-reperfusion injury in a chronic hypoxic rat model.

**KEYWORDS:** chronic hypoxia, ischemia-reperfusion injury, aging

## Original Article

**Age-Dependent Vulnerability to Ischemia-Reperfusion Injury of Cyanotic Myocardium in a Chronic Hypoxic Rat Model**Yasufumi Fujita\*, Kozo Ishino, Koji Nakanishi,  
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This study evaluated the effects of chronic hypoxia from birth on the resistance of rat hearts to global ischemia, with special emphasis on the duration of hypoxia. Male Wistar rats were housed from birth for 4 weeks or 8 weeks either in a hypoxic environment ( $\text{FiO}_2 = 0.12$ ) or in ambient air (8 animals for each group). Isolated rat hearts were perfused for 40 min with oxygenated Krebs-Henseleit buffer, subjected to 20 min global no-flow ischemia at  $37^\circ\text{C}$ , and then underwent 40 min of reperfusion. A non-elastic balloon was inserted into the left ventricle and inflated until the pre-ischemic LVEDP rose to 8 mmHg. Cardiac function was measured before and after ischemia. The post-ischemic percent recovery of LVDP in hypoxic hearts was worse than in normoxic hearts (4 weeks:  $55 \pm 7$  vs.  $96 \pm 3\%$ ,  $p < 0.01$ ; 8 weeks:  $40 \pm 5$  vs.  $92 \pm 4\%$ ,  $p < 0.01$ ), and was worst in the 8-week-hypoxic hearts. Similarly, the percent recovery of  $+dP/dt$  in the hypoxic hearts was lower than in the normoxic hearts (4 weeks:  $51 \pm 5$  vs.  $96 \pm 7\%$ ,  $p < 0.01$ ; 8 weeks:  $31 \pm 6$  vs.  $92 \pm 7\%$ ,  $p < 0.01$ ), and was lowest in the 8-week-hypoxic hearts. In conclusion, cyanotic myocardium revealed an age-dependent vulnerability to ischemia-reperfusion injury in a chronic hypoxic rat model.

**Key words:** chronic hypoxia, ischemia-reperfusion injury, aging

The hospital mortality rate associated with the repair of cyanotic congenital heart disease has decreased with recent advances in myocardial protection, surgical techniques, and postoperative intensive care [1, 2]. However, prolonged and/or complicated postoperative treatment is still extremely common for children treated for such a disease [3, 4].

Patients with adult congenital cyanotic heart disease often suffer from myocardial dysfunction after cardiac repair [5]. These patients frequently show

ultrastructural changes of the sort that are associated with severe degeneration and that are thought to correlate with clinical cardiac dysfunction [6].

Despite this clinical evidence, some experimental studies have concluded that chronic hypoxia has some cardioprotective effects [7]. Other studies have shown the opposite results. Throughout the debate, however, there has been little discussion of the duration of exposure to hypoxia [8]. We feel that experimental studies correlating the clinical evidence with the age of the patients could be useful.

To assist in laying the groundwork for such studies, this study was designed to evaluate the effects of chronic hypoxia from birth on the resistance of hearts

Received March 16, 2009; accepted May 18, 2009.

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to global ischemia, with a special emphasis on the duration of the hypoxia.

## Materials and Methods

**Animals.** Male Wistar rats were used in the current study. They were treated in compliance with the "Principles of Laboratory Animal Care" established by the National Society for Medical Research, and according to the principles contained in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Newborn litters of Wistar rats and their mothers were placed within 2 days of birth into either a hypoxic, normobaric chamber ( $\text{FiO}_2 = 0.12$ ) as described previously [9], or in a normoxic chamber. They were allowed free access to water and a chow diet until the start of the experiments. The rats were housed in their chambers either for 4 weeks or for 8 weeks, and 4 groups of animals were prepared.

**Assessment of ventricular function.** The experimental protocol is illustrated in Fig. 1.

All the rats were anesthetized with diethyl ether, and 1 unit- /BW (g) of heparin was injected. While under anesthesia, the animals were kept in an environment of the same oxygen concentration as that in which they had lived prior to surgery. Arterial blood samples were obtained from the exposed abdominal aorta. After a thoracotomy, the heart was rapidly excised and was arrested in cold Krebs-Henseleit bicarbonate buffer solution ( $4^\circ\text{C}$ ). The aorta was cannulated and Langendorff perfusion was initiated at a pressure of 100 cm  $\text{H}_2\text{O}$  with Krebs-Henseleit bicarbonate buffer solution, which contained 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.25 mmol/L  $\text{CaCl}_2$ , 25 mmol/L  $\text{NaHCO}_3$ , 1.2 mmol/L  $\text{MgSO}_4$ , 1.2 mmol/

L  $\text{KH}_2\text{PO}_4$ , and 11 mmol/L glucose, and was equilibrated with 95% oxygen and 5% carbon dioxide gas. The temperature of the perfusate was continuously monitored to assure that it was maintained at  $37.0 \pm 0.1^\circ\text{C}$ . The perfusate was filtered through  $5\mu\text{m}$  pores. The pulmonary artery was incised in order to permit drainage of the coronary sinus effluent. A non-elastic balloon filled with saline was inserted into the left ventricle through the atrium and connected by a catheter to a pressure transducer. Ten min after Langendorff perfusion was started, the balloon was inflated until the pre-ischemic LVEDP rose to 8 mmHg. The transducer was connected to a computer, and data were acquired with a PowerLab system (AD-Instruments, Grand Junction, CO, USA). Cardiac function was recorded, including the left ventricular developed pressure (LVDP), heart rate (HR), and the first derivatives of maximal rate of pressure over time ( $+dP/dt$ ). Coronary flow was measured by timed volumetric collection from the right side of the heart.

After allowing the contractile parameters to stabilize for 30 min, the pre-ischemic hemodynamic parameters were measured. Then, global warm ischemia ( $37^\circ\text{C}$ ) was induced for 20 min by clamping the aortic cannula. After the ischemic period, the heart was reperfused for 40 min at  $37^\circ\text{C}$  with the K-H buffer solution. The various indexes of cardiac function were measured again at this point.

At the end of this experiment, the hearts were removed from the apparatus, fixed in 10% buffered formalin for 24h, and then embedded in paraffin. Paraffin-embedded sections were cut and stained with hematoxylin and eosin stain for histopathological examination.

The heart was rejected when ventricular fibrillation continued over 20 min after reperfusion, or when the heart rate was less than 300 beats- /minute in

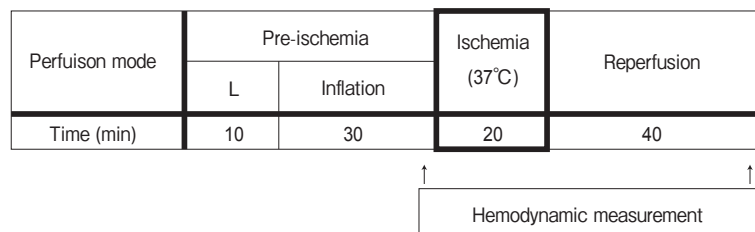


Fig. 1 Experimental protocol for ischemia-reperfusion study. L, Langendorff perfusion; Inflation, balloon inflation until pre-ischemic LVEDP rose to 8 mmHg.

4-week-old rats or less than 250 beats- /minute in 8-week-old rats.

**Statistical analysis.** All data were expressed as the mean  $\pm$  standard deviation. Differences between groups were first determined by a one-way-analysis of variance (ANOVA). Intergroup analysis was performed by using Fisher's PLSD test. A  $p$  value of less than 0.05 was considered to be statistically significant.

## Results

**Basal characteristics.** Table 1 shows the basal characteristics. The body weight of chronic hypoxic animals was lower than that of normoxic animals ( $p < 0.01$ ) in the groups of the same age. Arterial oxygen tension and oxygen saturation were lower in the hypoxic animals than in the normoxic animals ( $p < 0.01$ ), regardless of the age. Hypoxia resulted in a significant increase in the serum hemoglobin concentration and hematocrit ( $p < 0.01$ ).

**Pre-ischemic myocardial function.** Table 2 shows the pre-ischemic hemodynamic parameters. In

4-week-old rats, there was no difference in any of the parameters between the normoxic and hypoxic animals. In the 8-week-old rats, the LVDP and  $+dP/dt$  were significantly higher in the hypoxic animals ( $p < 0.01$ ); however, there was no difference in the heart rate between the normoxic and hypoxic animals.

**Post-ischemic myocardial function.** Table 3 shows the post-ischemic hemodynamic parameters. The percent recovery of LVDP in the hypoxic hearts was worse than in the normoxic hearts ( $p < 0.01$ ), and was worst in the 8-week-hypoxic hearts. Similarly, the percent recovery of  $+dP/dt$  in the hypoxic hearts was lower than in the normoxic hearts ( $p < 0.01$ ), and was lowest in the 8-week-hypoxic hearts. No difference was observed in the percent recovery of HR among the groups.

**Post-ischemic myocardial changes on light microscope.** Fig. 2 shows the post-ischemic changes on light microscope. Interstitial edema could be seen in all hearts; this change was more severe in the hypoxic hearts than in the normoxic hearts, and was worst in the 8-week-hypoxic rats. Little disruption of the myocardial cells was seen in the normoxic hearts,

Table 1 Basal characteristics

Variable	Group			
	4N	8N	4H	8H
Body weight (g)	104.3 $\pm$ 6.0	282.4 $\pm$ 11.2*	63.4 $\pm$ 5.9*	159.6 $\pm$ 19.3 <sup>+</sup>
Hemoglobin (g/dL)	13.9 $\pm$ 0.6	14.6 $\pm$ 0.8	18.3 $\pm$ 1.1*	20.3 $\pm$ 0.8 <sup>+</sup>
Hematocrit (%)	45.7 $\pm$ 3.4	47.4 $\pm$ 4.1	56.0 $\pm$ 5.6*	69.3 $\pm$ 5.6 <sup>+</sup>
PaO <sub>2</sub> (mmHg)	117.6 $\pm$ 1.5	116.2 $\pm$ 1.1	37.0 $\pm$ 3.4*	36.5 $\pm$ 4.3 <sup>+</sup>
O <sub>2</sub> sat (%)	97.5 $\pm$ 1.0	96.7 $\pm$ 0.7	68.9 $\pm$ 8.7*	67.2 $\pm$ 9.2 <sup>+</sup>

Data are expressed as the mean  $\pm$  standard deviation. N, normoxia; H, hypoxia; 4 or 8, 4-week-old or 8-week-old; PaO<sub>2</sub>, arterial oxygen tension; O<sub>2</sub> sat, oxygen saturation.

\* $p < 0.01$  4N vs. 8N, 4H, <sup>+</sup> $p < 0.01$  8N vs. 8H, \* $p < 0.01$  4H vs. 8H.

Table 2 Baseline measurements

Variable	Group			
	4N	8N	4H	8H
LVDP (mmHg)	121 $\pm$ 5	138 $\pm$ 3*	123 $\pm$ 3	149 $\pm$ 4 <sup>+</sup>
$+dP/dt$ (mmHg/s)	3,909 $\pm$ 368	3,708 $\pm$ 169	4,113 $\pm$ 433	4,306 $\pm$ 487 <sup>+</sup>
Coronary flow (mL/min)	7.9 $\pm$ 0.6	14.1 $\pm$ 2.6*	10.5 $\pm$ 0.6	21.3 $\pm$ 4.6 <sup>+</sup>
Heart rate (beats/min)	328 $\pm$ 25	299 $\pm$ 10*	329 $\pm$ 20	282 $\pm$ 18 <sup>+</sup>

Data are expressed as the mean  $\pm$  standard deviation. N, normoxia; H, hypoxia; 4 or 8, 4-week-old or 8-week-old; LVDP, left ventricular developed pressure;  $+dP/dt$ , the first derivatives of the maximal rate of pressure over time.

\* $p < 0.01$  4N vs. 8N, 4H, <sup>+</sup> $p < 0.01$  8N vs. 8H, \* $p < 0.01$  4H vs. 8H.

but damage was observed in the hypoxic hearts, and particularly in the 8-week-hypoxic rats.

## Discussion

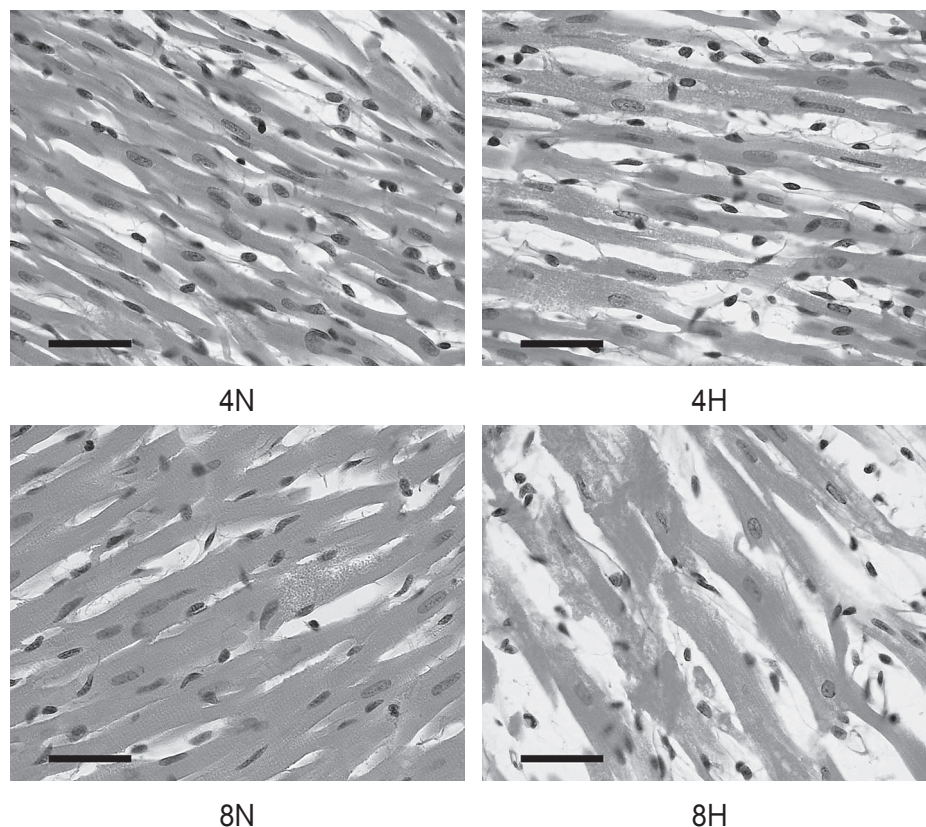
The present study demonstrated that chronic

**Table 3** Post-ischemic hemodynamic parameters

Variable	Group			
	4N	8N	4H	8H
LVDP (mmHg)	110 ± 8	127 ± 4*	68 ± 8*	64 ± 19 <sup>+</sup>
% recovery of LVDP	96 ± 3	92 ± 4	55 ± 7*	40 ± 5 <sup>+</sup> #
+dP/dt (mmHg/s)	3,733 ± 457	3,406 ± 266	2,098 ± 343*	1,336 ± 173 <sup>+</sup> #
% recovery of +dP/dt	95 ± 7	92 ± 7	51 ± 5*	31 ± 6 <sup>+</sup> #
Coronary flow (mL/min)	6.8 ± 0.6	11.2 ± 2.5*	6.8 ± 0.7	12.3 ± 2.3 <sup>+</sup>
% recovery of coronary flow	86 ± 3	79 ± 6*	65 ± 4*	58 ± 4 <sup>+</sup> #
Heart rate (beats/min)	313 ± 19	283 ± 18*	300 ± 18	258 ± 22
% recovery of HR	95 ± 3	95 ± 6	91 ± 3	92 ± 9

Data are expressed as the mean ± standard deviation. N, normoxia; H, hypoxia; 4 or 8, 4-week-old or 8-week-old; LVDP, left ventricular developed pressure; +dP/dt, the first derivatives of the maximal rate of pressure over time; HR, heart rate.

\* $p < 0.01$  4N vs. 8N, 4H, <sup>+</sup> $p < 0.01$  8N vs. 8H, # $p < 0.01$  4H vs. 8H.



**Fig. 2** Post-ischemic myocardial changes observed by light microscopy. Interstitial edema could be seen in all hearts. The change was worse in hypoxic hearts than in normoxic hearts, and was worst in 8-week-hypoxic rats. Little disruption of the myocardial cells was seen in normoxic hearts, but damage was observed in hypoxic hearts, particularly in 8-week-hypoxic rats (hematoxylin and eosin stain; scale bar, 100  $\mu$ m). N, normoxia; H, hypoxia; 4 or 8, 4-week-old or 8-week-old.



hypoxia from birth impaired the post-ischemic recovery of cardiac function in hearts that were subjected to 20 min of warm global ischemia. The impairment was worse when the duration of the exposure to chronic hypoxia was longer. The duration of hypoxia exposure also affected the degree of interstitial edema and the degree of disruption of the myocardial cells after ischemia-reperfusion.

Some reports suggest that developing mammalian hearts are more resistant to the effects of cardiac insults than adult hearts [10–13]. Imura *et al.* reported that the extent of myocardial protection with cold-crystalloid in pediatric open heart surgery was dependent on the patient's age and degree of cyanosis [14]. However, there are few experimental studies that address the effects of the duration of exposure to chronic hypoxia upon ischemia-reperfusion injury. It is our hope that the current study may have demonstrated clinically-relevant results about the effects of the duration of exposure to hypoxia.

The test group in the current study was exposed to normobaric chronic hypoxia from birth. The method used to create chronic hypoxia was simple and noninvasive, and by it we were able to avoid the mixture of outside air with the air in the chamber. The selected  $\text{FiO}_2$  was 0.12, and the hemoglobin, hematocrit, arterial oxygen tension, and oxygen saturation observed in the hypoxic rats were similar to those observed in patients with congenital cyanotic heart diseases [15]. The increase of hemoglobin and hematocrit are due to increased hypoxia-induced erythropoietin production [16].

We found no difference between the pre-ischemic left ventricular functioning of the 4-week-old hearts, whether normoxic or hypoxic, but the functioning was significantly higher in the 8-week-hypoxic hearts than in the 8-week-normoxic hearts. In contrast, Najm *et al.* reported that children with congenital cyanotic heart diseases show a lower ejection fraction than acyanotic children, and that this was because the hearts of children with cyanosis have lower ATP levels than those of acyanotic children [3]. This discrepancy might be explained by the fact that children with cyanosis have never been exposed to normoxia, even when they are anesthetized, until surgical correction is performed, but the rats in this study were exposed to a higher oxygen environment during the pre-ischemic state, and this could have increased the ATP

content of their hearts. Samanek *et al.* reported that the levels of energy-supplying enzymes, such as hexokinase, triosephosphate dehydrogenase, lactate dehydrogenase, and so on in the right ventricles of children with cyanotic heart diseases were no different from those of acyanotic children, with the exception of citrate synthase, which was decreased in cyanotic children [17]. This result suggests that, in the current experiment, exposure to a higher oxygen concentration may have increased the ATP content of the hypoxic hearts more greatly than it increased the ATP content of the normoxic hearts.

The post-ischemic recovery of cardiac function was impaired in the hypoxic hearts, especially in the older hearts. Both severe interstitial edema and destruction of myocardial cells were more common in the hypoxic hearts than in the normoxic hearts, and these characteristics of course contributed to the impairment of cardiac function. As mentioned, the edema and the cell destruction were even more severe in the older hypoxic hearts than in the younger hypoxic hearts. These results suggest that the hearts from hypoxic animals appear more vulnerable than normoxic hearts to ischemia-reperfusion injury, and that the longer the exposure to hypoxia, the lower the functional recovery of the hearts after ischemia-reperfusion. The cause of these results could not be determined in the current experiment, but a hypothesis may be considered. In a previous study, after 8-week-old rats were housed either in a hypoxic or in a normoxic environment for 2 weeks, mitochondrial superoxide dismutase, cytosolic reduced glutathione, and mitochondrial and cytosolic glutathione reductase activity were significantly lower in the hypoxic animals than in the normoxic animals at end-ischemia [9]. These results suggest that chronic hypoxia may inhibit or delay the metabolic maturation of antioxidant systems. This inhibition may be serious for immature hearts. The hypothesis, therefore, is that chronic hypoxia from birth may inhibit the metabolic maturation of antioxidant systems and that the antioxidant reserve may be more reduced as the duration of exposure to hypoxia increases. Further studies, such as the direct detection of free radicals or the administration of free radical scavengers, are required to prove this hypothesis.

The current results are not consistent with results obtained with chronically hypoxic rabbit hearts, which were found to be more tolerant of ischemia-reperfu-

sion injury than normoxic hearts [7]. This discrepancy may simply result from the difference in the species. Clinical reports have shown that cyanotic children have poor outcomes, such as postoperative cardiac dysfunction, and more reperfusion injury in comparison to acyanotic children [3, 4]. Therefore, the current study may be more clinically-relevant than the models used in other studies.

The current results also differ from those observed with chronically hypoxic rat hearts that were exposed to low air pressure or intermittent hypoxia [18, 19]. However, these methods can never avoid reoxygenation, even if it is for the very short periods necessary for maintenance of the cages. Children with congenital cyanotic heart diseases are never exposed to normoxia until surgical corrections are performed, and the effects of short-time reoxygenation on heart development in such children is not known. The rats in the current study were not exposed to normoxia until the experiment after they were housed in a hypoxic environment, and might be similar to cyanotic children.

The model used in this study has several limitations. First, the experiments used an isolated perfused preparation. The preparation is denervated [20]. However, an advantage is that direct cardiac responses can be studied independent of various factors. Second, we chose to use a crystalloid solution in the perfusion circuit because, with blood perfusion, each blood component serves different roles during ischemia and reperfusion and could have confused the results. Nonetheless, blood perfusion may have induced different results from those of the crystalloid perfusion [21].

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